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Phylogeny of Siberian species of *Carex* sect. *Vesicariae* based on nuclear and plastid markers

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Carex sect. *Vesicariae* is a group of about 30 sedge species widespread in temperate and cold regions of Eurasia and North America. In this study we performed a phylogenetic analysis of ten taxa (9 species and one subspecies) found in Siberia based on two plastid sequences (*matK* and *atpF-H*) and the nuclear ribosomal ITS2 sequence. We also developed a novel sequence marker for the genus *Carex*, an intron of the nuclear *hsp90* gene, which was found to be several times more variable than plastid and nuclear ribosomal sequences. Total nucleotide variation within the *Vesicariae* section was found to be very low, comparable to that within single other sedge species. Phylogenetic analysis supported the existence of two groups within this section: clade A (*C. vesicata*, *C. vesicaria*, both subspecies of *C. saxatilis* and *C. pamirica*) and clade B (*C. rostrata*, *C. mollissima*, *C. jacutica*, *C. rotundata* and *C. membranacea*). *Carex rhynchophysa* holds an intermediate position between these groups. The main morphological difference between these clades lies in the nature of the transition of the utricle into the beak: it is gradual in clade A and abrupt in clade B. This division is in good accordance with the results of previous allozyme studies and with current taxonomy. These results may become the basis for consideration of subsectional taxa within this section.

The genus *Carex* L. (family Cyperaceae Juss.) is one of the largest and most widespread genera of flowering plants; it includes over 2000 species (Reznicek 1990). For many sections of this genus, morphological differences between species are often subtle, which makes it hard to reconstruct their phylogenetic relationship using morphological data alone. This situation is complicated by the existence of a great number of interspecific hybrids, which have intermediate habitus in comparison to parent species and may be fertile or partly fertile (Cayouette and Catling 1992, Egorova 1999). In the last decade, molecular genetic markers have proven to be very useful for studying the relationships of species and subgeneric taxa within the genus *Carex* (Roalson et al. 2001, Heindrichs et al. 2004, Waterway and Starr 2007, Waterway et al. 2010).

The section *Vesicariae* Meinsh. is a member of subgenus *Carex*; it contains over 30 species and subspecies (Mackenzie 1935, Chater 1980, Kozhevnikov 1988, Egorova 1999, Ball and Reznicek 2002, Dai et al. 2010). Species of this section are widespread in temperate and cold regions of Eurasia and North America, usually in swamp and waterside habitats. Ten species and subspecies are found in Siberia (Malyshev 1990).

Species belonging to the section *Vesicariae* have the following characteristics: 1) 1–5 upper spikes are male, and the other 2–5 spikes are female, 2) the lowest bract is sheath-less; blade is longer or equal to the inflorescence, 3) utricles are inflated, bulliform membranaceous or thin-coriaceous, usually ovoid, 3–8 mm in length; the beak of the utricule is generally elongate, bidentate (more rarely, emarginate or entire), 4) 2–3 stigmas are present, 5) rhizomes are creeping (Egorova 1999).

Most taxonomists have not formally recognized subsections or other groups within section *Vesicariae* (Mackenzie 1935, Reznicek 1990, Egorova 1999, Reznicek and Ford 2002, Dai et al. 2010). This section is sometimes merged with the sect. *Pseudocypereae* Tucker. ex Kük. (Kreczetowicz 1935, Ball and Reznicek 2002) and/or sect. *Carex* L. (Kreczetowicz 1935). Ford et al. (1991, 1993) and Ford and Ball (1992) divided this section into two informal species groups, the short-beaked and long-beaked taxa. The short-beaked species (*C. saxatilis* L., *C. membranacea* Hook., and *C. rotundata* Wahlenb.) are characterized by arctic or high boreal distribution, weakly nerved perigynia, and short beaks (<1 mm long) that are indistinctly toothed at the apex, while the long-beaked species (*C. rostrata* Stokes, *C. vesicaria* L. and *C. utriculata* Boott) have a more temperate distribution, prominently nerved perigynia, and long beaks (>1 mm long) that are distinctly toothed at the apex. This division, however, was not well supported by allozyme analysis: species of the long-beaked and short-beaked groups

were intermixed and the authors concluded that these informal groups are not monophyletic (Ford et al. 1993).

Kreczetowicz (1935) combined species of the sect. *Vesicariae* together with the sectt. *Carex*, *Rostrales* Meinsh. and *Pseudocypereae* into a large sect. *Pompholyx* V. I. Krecz. We should note that the names of the cycles and rows specified by Kreczetowicz mentioned below are invalid according to the paragraph 36.1 of the 'International code of botanical nomenclature' (McNeill et al. 2006), because their descriptions do not include Latin diagnosis. This section is divided into five cycles, and the species of the section *Vesicariae* are divided between two cycles:

cycle *Ampullaria* V. I. Krecz.
- *C. rhynchophysa* C. A. Mey.
- *C. jacutica* V. I. Krecz.
- *C. inflata* Huds.
- *C. stenolepis* Less.
- *C. rotundata*
- *C. utriculata*

cycle *Vesicularia* V. I. Krecz.
- row *Chlorostachyae* V. I. Krecz.
  - *C. vesicaria*
  - *C. vesicata* Meinsh.
- row *Poecilostachyae* V. I. Krecz.
  - *C. grahamii* Boott.
  - *C. dichroa* (Freyn) V. I. Krecz. (sine auct. comb.)
  - *C. pamirensis* C. B. Clarke (priority name *C. pamirica* (O. Fedtsch.) O. et B. Fedtsch.))
- row *Melanostachyae* V. I. Krecz.
  - *C. saxatilis*
  - *C. procerula* V. I. Krecz.
  - *C. membranacea*

These cycles were divided based on the nature of the transition between the utricle and the beak (this transition is abrupt in the species of the cycle *Ampullaria* and gradual in the cycle *Vesicularia*). Kreczetowicz (1935) treated *C. mollissima* Christ as belonging to a separate sect. *Malacocarex* V. I. Krecz. together with *C. planiculmis* Kom. (currently included into sect. *Anomalae* (Carey) Mackenzie) based on inflorescence characters (upper spikelet is staminal, other 3–5 flowers are pistillate; spikelets are pedunculate, standing or drooping); however, other taxonomists have not accepted this treatment.

In this study, the section *Vesicariae* is treated according to Egorova (1999). The following species and subspecies found in Siberia were studied: *C. rostrata*, *C. rhynchophysa*, *C. jacutica*, *C. rotundata*, *C. vesicaria*, *C. vesicata*, *C. pamirica* subsp. *pamirica*, *C. pamirica* subsp. *dichroa* (Freyn) T. V. Egorova, *C. saxatilis* L. subsp. *laxa* (Trautv.) Kalela, and *C. mollissima*. In addition, we also sampled *C. capricornis* Meinsh. ex Maxim. and *C. pseudocyperus* L. belonging to the closely related sect. *Pseudocypereae* as outgroup.

The aims of this work were 1) to study genetic variation within the species of the section *Vesicariae* using plastid and nuclear sequences, including a novel nuclear marker, the intron of the *hsp90* gene developed by us, and 2) to reconstruct phylogenetic relationships in this section using sequence data.

Material and methods

Herbarium specimens of the species of *Carex* sect. *Vesicariae* were taken from the NSK Herbarium. For each taxon, we sampled 3–7 specimens from distant geographic locations (except for outgroup species). Details on the specimens are given in Table 1.

Total DNA was extracted from 10–100 mg of dried leaves. Tissues were ground with sterile sand using a mortar and a pestle and incubated 4 h at 65°C in a buffer containing 3% CTAB, 1.4 M NaCl, 30 mM Tris-HCl (pH = 8.0), and 2 mM EDTA. DNA was extracted with chloroform and precipitated with isopropyl alcohol. After precipitation, DNA was dissolved in distilled water and purified on silica columns according to the manufacturer's instructions. DNA obtained using this method allowed us to reliably amplify sequences from specimens up to 50 years old (the oldest specimen in this study was collected in 1958).

PCRs were performed in a 25 μl volume reaction containing 1.5 mM $MgCl_2$, 65 mM Tris-HCl (pH = 8.8), 16 mM $(NH_4)_2SO_4$, 0.05% Tween-20, 0.2 mM of each dNTP, 0.3 mM primers, and 1 unit of recombinant Taq polymerase.

Because DNA extracted from herbarium specimens is usually highly fragmented, we designed primers so that the resulting fragment was no longer than 600 bp. Primers for ITS2 (CITS2-F2, 5′-CAACG-GATAT-CTCGG-CTC TC-3′, CITS2-R2, 5′-GATTC-GCTCG-CCGTT-ACT AT-3′) and *matk* fragment (central portion of the *matk* gene) (matK-1, 5′-TTCAA-ATCCT-TCAAT-GCTGG-3′, matK-3, 5′-TGAGA-GGAAG-GACTG-GAACT-AA-3′) were designed using the sequences of *matK* and the nuclear ribosomal cluster of various *Carex* species obtained from GenBank. Primers for the *atpF-H* intergenic spacer were adopted from Fazekas et al. (2008). Because there was a significant difference of melting temperature between these primers, we designed a nested forward primer (AtpF2, 5′-CCCAA-GAAAA-CGAAA-GAATC-3′).

Primers for the *hsp90* gene have been reported to amplify a fragment of a presumable single-copy heat-shock protein gene in various plant taxa (Steele et al. 2008). The original primers from that study (*hsp90*-Fw, 5′-ACGGA-CAAGA-GCAAG-CTCGA-TG-3′, *hsp90*-Rv, 5′-TTGTA-GTCTT-CCTTG-TTCTC-AG-3′) amplified a fragment about 1500 bp long, which contained an intron about 400 bp long. This intron turned out to be rather variable, and specific primers amplifying a 3′ part of this intron were designed (HSP-s1, 5′-TGACC-CTTTA-CCTCA-AGGAT-G-3′, HSP-s2, 5′-GCGCT-CCTCA-AGATA-CTCCA-3′). In some cases the resulting amplicon was a mixture of sequences of different length as the result of length polymorphism in a poly-T sequence. For some specimens, the *hsp90i* amplicon was cloned into pBluescript and several clones for each specimen were sequenced; for others, sequences read from both primers were concatenated in the poly-T region. All sequences obtained in this study were deposited in GenBank under accession no. JN314437–JN314629 (Table 1).

Phylogenetic trees using minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) algorithms were constructed using the MEGA program (Tamura et al. 2011). Branch support was calculated using

Table 1. Collection data for the studied specimens. v. = village, t. = town, r. = river, l. = lake, NR = nature reserve.

Specimen no.	Species	Sampling locatily	Collection date	Collectors	Genbank accession no.			
					ITS2	*matK*	*atpF-H*	*hsp90i*
C101	*C. rostrata*	Tuva, Erzin region, near v. Naryn, flood plain of r. Naryn. h = 1200 m	10 Jul 1972	I. Krasnoborov, L. Kosinets	JN314569	JN314484	JN314477	JN314617
C102	*C. rostrata*	Buryatia, Pribaikal region, l. Baikal, v. Turka, source r. Bezymyannaya	198?	V. Doronkin		JN314485	JN314478	
C103	*C. rostrata*	Irkutsk oblast, Mamsko-Chuiskii region, right bank of r. Vitim, 15 km from t. Mama	17 Jul 1977	M. Ivanova, V. Zuev	JN314568	JN314486	JN314479	JN314618
C104	*C. rostrata*	Novosibirsk oblast, Maslyanino region, near v. Aleksandrovka, swamp Klyukvennoye	19 Jul 1989	Volkova	JN314554	JN314487	JN314480	
C048	*C. rostrata*	Khabarovsk krai, Verkhnebureinskii region, near v. Sophiisk, valley of r. Olga	10 Sep 1978	A. A. Nechaev	JN314556	JN314530		JN314619
C105	*C. rhynchophysa*	Irkutsk oblast, Zhigalovskii region, near v. Yakimovka	28 Jun 1961	Balochanov	JN314549	JN314488	JN314460	JN314614
C106	*C. rhynchophysa*	Irkutsk oblast, Chunskii region, upper reaches of r. Kova	28 Aug 1958	E. M. Zlobina	JN314555	JN314489	JN314461	
C107	*C. rhynchophysa*	Buryatia, Tunkin region, near v. Zun-Murino	14 Jul 1962	N. Lebedishchev	JN314550	JN314490	JN314462	JN314615
C108	*C. rhynchophysa*	Buryatia, Muya valley	28 Jul 1977	A. Chepurnov	JN314552	JN314491	JN314463	JN314616
C109	*C. rhynchophysa*	Yakutia, Aldan region, 6 km downstream from t. Malyi Nimnyr	21 Jul 1982	N. Bolshakov, V. Telegin	JN314551	JN314492	JN314464	
C110	*C. rhynchophysa*	Yakutia, Aldan region, near t. Malyi Nimnyr	13 Jul 1982	N. Kovtonyuk, V. Telegin	JN314553	JN314493	JN314465	JN314613
C096	*C. rhynchophysa*	Tuva, Pii-Khem region. flood plain of r. Bilepig. h = 1657 m, 52021′14″N, 54056′20″E	17 Jul 2010	I. V. Khan, E. A. Balde		JN314533	JN314476	JN314612
C113	*C. jacutica* × *?*	Yakutia, Olenek region, r. Arga-Salaa	24 Jul 1978	N. Vodopyanova	JN314566	JN314496	JN314467	JN314600, JN314608
C114	*C. jacutica*	Yakutia, Verkhnekolymsk region, Momskii ridge, 120 km to SE from t. Zyryanka. h = 400 m	13 Jul 1983	N. Bolshakov, A. Vanyaev		JN314497	JN314468	
C115	*C. jacutica*	Yakutia, Tompon region, road Khandyga-Magadan, r. Tomnopuk	7 Jul 1984	O. Nikiforova	JN314565	JN314498	JN314469	JN314623
C122	*C. jacutica*	Yakutia, Neryungri region, near t. Nagornyui, left bank of r. Timpton	30 Jun 1982	N. Bolshakov, N. Vlasova	JN314573	JN314503	JN314474	JN314627
C118	*C. mollissima*	Buryatia, Stanovoye plateau,Yuzhno-Muiskii ridge, upper reaches of r. Gorbylok-Muiskii	10 Aug 1965	V. Burkova, A. Naumov	JN314564	JN314499	JN314470	JN314624
C119	*C. mollissima*	Zabaykalskii krai, Chernyshevskii region, r. Kuenga, tributary of the r. Chilka, v. Ust-Gorbitsa	25 Jun 1964	G. Peshkova, L. Turova	JN314563	JN314500	JN314471	JN314625
C080	*C. mollissima*	Khabarovsk krai, Verkhnebureinskii region, near v. Sophiisk, valley of r. Olga	2 Aug 1978	A. A. Nechaev	JN314570	JN314532		JN314626
C120	*C. rotundata*	Tyumen oblast, right bank of r. Ob, mount Tugiyanovskaya	13 Aug 1986	L. Malyshev	JN314562	JN314501	JN314472	
C121	*C. rotundata*	Irkutsk oblast, basin of r. Kotui, l. Essei	9 Aug 1973	S. Andrulaitis		JN314502	JN314473	JN314628
C112	*C. rotundata*	Yakutia, Udokan ridge, left tributary of r. Chitkanda. h = 1450 m	8 Aug ????	N. Vodopyanova, T. Kobylkina	JN314567	JN314495	JN314466	JN314629
C123	*C. rotundata*	Yuzhno-Muiskii ridge, left river head of r. Mogoi. h = 1740 m	26 Jul 1966	M. Ivanova, V. Suslov			JN314475	
C124	*C. vesicaria*	Kurgan oblast, Shchuchie region, near v. Dan'kovo	14 Jun 1984	N. Bolshakov, V. Doronkin		JN314504	JN314442	
C125	*C. vesicaria*	Tyumen oblast, Golyushmanovo region, v. Skarednoye	14 Jul 1984	A. Krasnikov, V. Zuev	JN314544	JN314505	JN314439	JN314610

(continued)

Table 1. (Continued)

Specimen no.	Species	Sampling locatily	Collection date	Collectors	Genbank accession no.			
					ITS2	*matK*	*atpF-H*	*hsp90i*
C127	*C. vesicaria*	Kemerovo oblast, Novokuznetsk region	15 Jul 1986	M. Revyakina	JN314541	JN314506	JN314440	JN314602
C128	*C. vesicaria*	Irkutsk oblast, Kazachinsk region, v. Konets Lug	31 Jul 1976	N. Vodopyanova		JN314507	JN314445	JN314611
C052	*C. vesicaria*	Krasnoyarsk krai, Motygino region, v. Motygino, right bank of r. Angara	1 Jul 1981	A. Chepurnov	JN314540	JN314601	JN314531	
C129	*C. vesicata*	Buryatia, Ulan-Ude city, r. Selenga	15 Jun 1997	L. Malyshev	JN314538	JN314508	JN314441	JN314605
C130	*C. vesicata*	Buryatia, Bargusin region, l. Baikal, Bargusin bay	29 Jul 1987	N. Vlasova	JN314536	JN314509	JN314438	JN314604
C131	*C. vesicata*	Irkutsk oblast, Bodaibo region, Vitim NR, r. Vitim	28 Aug 1986	A. Kiseleva	JN314535	JN314510	JN314446	JN314598–JN314599, JN314609
C132	*C. vesicata*	Yakutia, Srednekolymsk region, near t. Lobui, right bank of r. Kolyma	30 Aug 1983	N. Bolshakov, A. Vanyaev	JN314537	JN314511	JN314447	JN314603
C111	*C. vesicata*	Irkutsk oblast, Kachug region, l. Ochaul	26 Jul 1976	N. Vodopyanova	JN314545	JN314494	JN314437	JN314581–JN314583, JN314606–JN314607
C133	*C. saxatilis* subsp. *laxa*	Krasnoyarsk krai, Putorana plateau	18 Jul 1970	N. Vodopyanova	JN314547	JN314512	JN314448	JN314595
C134	*C. saxatilis* subsp. *laxa*	Krasnoyarsk krai, Putorana plateau, h = 650 m	11 Aug 1969	N. Vodopyanova	JN314548	JN314513	JN314449	JN314591
C135	*C. saxatilis* subsp. *laxa*	Tuva, Erzin region, Sangilen plateau, upper reaches of r. Balyktyk-Khem, near l. Dakhuu-Nur. h = 2220 m	17 Jul 1973	I. Krasnoborov, V. Lebedev	JN314561	JN314514	JN314450	JN314592
C001	*C. saxatilis* subsp. *laxa*	Buryatia, Okinskiy region, l. Ilchir, source of r. Irkut, left bank. h = 1954 m, 51059′30.7″N, 101000′43.1″E	21 Aug 2007	I. N. Shekhovtsova, A. A. Petruk	JN314534	JN314526	JN314451	JN314593, JN314594
C042	*C. saxatilis* subsp. *laxa*	Krasnoyarsk krai/Taymyr boundary, basin of r. Khatanga, near v. Kresty	8 Aug 1974	S. Andrulaitis	JN314572	JN314528	JN314443	JN314596
C043	*C. saxatilis* subsp. *laxa*	Yakutia, Verkhnekolymsk region, near v. Zyryanka	29 Jun 1983	N. Bolshakov, A. Vanyaev	JN314571	JN314529		
C136	*C. pamirica* subsp. *pamirica*	Buryatia, Tunkin region, 40 km from t. Mondy, upstream of r. Irkut, right bank. h = 1886 m, 51054′53.3″N, 100046′05.2″E	19 Aug 2007	I. N. Shekhovtsova, A. A. Petruk	JN314560	JN314515	JN314453	
C137	*C. pamirica* subsp. *pamirica*	Buryatia, Tunkin region, 40 km from t. Mondy, upstream of r. Irkut, right bank. h = 1886 m, 51054′53.3″N, 100046′05.2″E	19 Aug 2007	I. N. Shekhovtsova, A. A. Petruk	JN314559	JN314516	JN314454	
C138	*C. pamirica* subsp. *pamirica*	Altai Republic, Kosh-Agach region, Ukok plateau, right bank of r. Zhumaly. h = 2108 m, 49033′57.1″N, 87057′55.8″E	18 Aug 2006	A. A. Petruk, I. N. Shekhovtsova	JN314546	JN314517	JN314455	JN314577
C139	*C. pamirica* subsp. *pamirica*	Buryatia, Okinskii region, l. Ilchir, source of r. Irkut, left bank. h = 1954 m, 51059′30.7″N, 101000′43.1″E	21 Aug 2006	I. N. Shekhovtsova, A. A. Petruk	JN314558	JN314518	JN314456	JN314589
C140	*C. pamirica* subsp. *pamirica*	Irkutsk oblast, Olkhon region, Pribaikal NR, Pimorskii ridge, Eastern shore of l. Baikal, source of the Sarma river. h = 453 m, 53005′53.8″N, 106051′41.5″E	17 Jul 2007	I. N. Shekhovtsova	JN314542	JN314519	JN314457	JN314587

C141	*C. pamirica* subsp. *pamirica*	Buryatia, Okinskii region, l. Ilchir, source of r. Irkut, left bank. h = 1954 m, 51059′30.7″N, 101000′43.1″E	21 Aug 2007	I. N. Shekhovtsova, A. A. Petruk	JN314557	JN314520	JN314458	JN314590
C142	*C. pamirica* subsp. *dichroa*	Irkutsk oblast, Slyudyanka region, t. Kultun. h = 476 m, 51042′59″N, 103042′30″E	1 Aug 2010	S. G. Kazanovskii, S. V. Ovchinnikova	JN314543	JN314521	JN314459	JN314588
C004	*C. pamirica* subsp. *dichroa*	Irkutsk oblast, Olkhon region, Pribaikal NR, Primorskii ridge, eastern shore of l. Baikal, source of r. Sarma. h = 453 m, 53005′59.1″N, 106051′41.5″E	17 Jul 2007	I. N. Shekhovtsova, A. A. Petruk	JN314539	JN314527	JN314452	JN314578–JN314580, JN314584–JN314586, JN314597
C143	*C. capricornis*	Buryatia, Kyakhta region, 4 km from v. Polkanovo, l. Rybnoye	11 Jul 2001	O. Anenkhonov	JN314574	JN314522	JN314481	JN314622
C144	*C. pseudocyperus*	Omsk oblast, Shcherbakul region, l. Gladkoe	26 Jun 1984	S. Bubnova	JN314575	JN314523	JN314482	JN314620
C146	*C. pseudocyperus*	Kemerovo oblast, Promyshlennaya region, near t. Zhuravlevo	11 Jul 1986	I. Makhotkov, N. Lashchinskii	JN314576	JN314525	JN314483	JN314621

the bootstrap test; 1000 replications were performed for each algorithm. Evolutionary distances for ME were computed using the maximum composite likelihood model. MP trees were obtained using the close-neighbor-interchange (CNI) algorithm. The Tamura–Nei 3-parameter model was used for ML. Bayesian analysis was performed using MrBayes (Ronquist and Huelsenbeck 2003). Two simultaneous independent analyses were run from different random starting trees using four chains of 'metropolis coupled Monte Carlo' simulations for 1 000 000 generations, sampling a tree every 100 generations; the first 25% of trees of each run were discarded. The following substitution models were chosen by MrModeltest (Nylander 2004) based on hierarchical likelihood ratio tests: HKY for *hsp90i* and *matk*, HKY + I for ITS2, and F81 for *atpF-H*.

Results

ITS2 variation

The ribosomal ITS2 sequence is one of the most widely used in phylogenetic studies (Schultz and Wolf 2009). Within this sequence we found ten variable positions in 41 studied sequences, five of them parsimony informative (Table 2). However, for all these five sites sequences with heterozygous positions were observed; as the result, no clades were identified in the ITS2 tree (not shown). Sequence heterozygosity may be the result of interspecific hybridization, of coexistence of rRNA variants within individuals, or be due to incomplete lineage sorting. We conclude that ITS2 seems to be of little use for barcoding or phylogeny reconstruction purposes within the section *Vesicariae*.

atpF-H spacer variation

We found only one nucleotide substitution and three indels in the studied 499 bp sequence. A 8 bp long insertion was characteristic for specimens of *C. rostrata*; two insertions 7 and 9 bp long were found in the C042 *C. saxatilis* subsp. *laxa* and C130 *C. vesicata* specimens, respectively. The only substitution (G<>A in the 454 position) divided our sample in two parts: a group containing *C. vesicata*, *C. vesicaria*, *C. saxatilis* subsp. *laxa*, and both subspecies of *C. pamirica* (further referred to as clade A; below) and a group comprising *C. mollissima*, *C. jacutica*, *C. rotundata*, and *C. rostrata* (clade B) plus *C. rhynchophysa*. Sequences of *C. saxatilis* subsp. *saxatilis* from other studies from GenBank (FJ548447, FJ548449–FJ548451) fell into the first group,

Table 2. Sequence variation of the studied molecular markers within the section *Vesicariae*.

	atpF-H	*matK*	ITS2	*hsp90i*
Sequence length	490–500	591	438	314–366
Number of variable sites	1	12	10	31
Number of parsimony-informative sites	1	3	5	11
Number of indels	3	–	–	5
Number of sequences sampled	47	44	41	35

and *C. membranacea* accessions (FJ548426–FJ548432) fell into the second group.

matK variation

One of the three parsimony-informative sites (G>A substitution in the position 60) was characteristic for *C. mollissima* specimens; a G<>T substitution in position 502 again divided our sample into the same groups as mentioned above; and one C>T substitution was characteristic for *C. rhynchophysa*, *C. jacutica*, and all but one *C. rotundata* specimens. Again the *C. saxatilis* subsp. *saxatilis* GenBank sequences of other authors (FN668460, FJ548136) fell into clade A; *C. membranacea* GenBank sequences (FJ548112, FJ548114) were identical to those of *C. rostrata* and *C. mollissima*.

Thus, the plastid *matK* and *atpF-H* sequences are also characterized by low sequence diversity, and we conclude that none of these three generally used sequences can be used for species identification, except for a few species.

hsp90i variation

In addition to these three sequences, we developed a novel sequence marker, the *hsp90i* intron sequence. Sequences variation in *hsp90i* was more than in ITS2 and plastid markers taken together (Table 2). The main disadvantage of this sequence marker is length polymorphism in the central poly-T stretch in about a third of the studied specimens, which forced us to clone the amplicons.

Sequences of *hsp90i* of clades A and B differed by six nucleotide substitutions and two indels (Table 3). Within clade B, all species could be reliably identified by combinations of characteristic indels and/or substitutions (Table 3). In contrast, almost no differences were found within clade A; one substitution (C>T in 348 position) was characteristic for *C. saxatilis* subsp. *laxa*; and there were no characteristic substitutions among *C. vesicaria*, *C. vesicata*, and both subspecies of *C. pamirica*. *C. rhynchophysa* grouped together with clade A: it shared all characteristic substitutions and indels with it, but had two additional substitutions in the positions 342 and 357 that distinguished it from both clades A and B.

Discussion

Sequence variation within the section *Vesicariae*

Our results demonstrate that the overall sequence variation within the section *Vesicariae* is very low; in fact, species of clade A were indistinguishable even using all four markers. Even both plastid sequences of the outgroup species *C. pseudocyperus* and *C. capricornis* differed from the species of sect. *Vesicariae* section by only 3 and 4 nucleotide substitutions, respectively. This low variation is especially striking if we compare it to intraspecific variation in other *Carex* species. For example, Yano et al. (2010) found 28 substitutions and 16 haplotypes in ~2000 bp of plastid sequences of the *C. conica* Boott complex. King and Roalson (2009) found 57 different haplotypes in an *rpL16* plastid gene fragment

Table 3. Characteristic substitutions and indels within the studied species of *Carex* sect. *Vesicariae*. [1] = TCTTATAG, [2] = GATA, [3] = A, [4] = AAATTTA, [5] = GAACTAGACATCATGCATTTTTAGTCTTGATTA GATAAATGTG, [6] = GTCCATGTT.

	matk			*atpF-H*		*hsp90i*											
Species	60	318	502	33	454	36	37	57	111	121	202	213	227	246	291	295	342	346	348	357	359
C. rhynchophysa	G	C	T	–	A	T	–	–	A	ins[4]	T	G	–	T	C	C	C	–	C	A	A
Clade A																					
C. vesicaria	G	T	G	–	G	T	–	–	A	ins	T	G	–	T	C	C	T	–	C	G	G
C. vesicata	G	T	G	–	G	T	–	–	A	ins	T	G	–	T	C	C	T	–	C	G	G
C. pamirica subsp. *pamirica*	G	T	G	–	G	T	–	–	A	ins	T	G	–	T	C	C	T	–	C	G	G
C. pamirica subsp. *dichroa*	G	T	G	–	G	T	–	–	A	ins	T	G	–	T	C	C	T	–	C	G	G
C. saxatilis subsp. *laxa*	G	T	G	–	G	T	–	–	A	ins	T	G	–	T	C	C	T	–	T/Y	G	G
Clade B																					
C. rostrata	G	T	T	ins[1]	A	T	–	ins[3]	C	–	C	A/R	del[5]	–	T	T	T	–	C	G	A
C. rotundata	G	C/T	T	–	A	T	–	ins	C	–	C	G	–	G	T	T	T	del[6]	C	G	A
C. mollissima	A	T	T	–	A	A	ins[2]	ins	C	–	C	G	–	G	T	T	T	–	C	G	A
C. jacutica	G	C	T	–	A	A	–	ins	C	–	C	G	–	G	T	T	T	–	C	G	A

756 bp long in North American *C. macrocephala* Willd. ex Spreng. Other studies reported lower intraspecific variation, varying from 2 to 10 substitutions within different plastid markers (Senni et al. 2005, Schönswetter et al. 2006, Puşcaş et al. 2008), which is comparable with sequence variation within the whole *Vesicariae* section.

Interspecific hybrids

There is a widely supported opinion that the genus *Carex* is characterized by numerous interspecific hybrids (Cayouette and Catling 1992, Egorova 1999). We have found one clear case of hybridization between the A and B clades, the C113 specimen originally identified as *C. jacutica*. Its plastid haplotypes belong to the B clade, and its nuclear *hsp90i* sequences to the A clade. On the whole, this specimen is morphologically most similar to *C. jacutica*: its stems are 30 cm high, there are 3 motley-brown male spikes 1–2 cm long and 3 female spikes 0.7–2.0 cm long, oblong or ovoid, subsessile, or the lowest spike having a short peduncle up to 2 cm long. The lowest bract is shorter than the inflorescence. Utricules are reverse ovoid, swollen, 4.0–4.5 mm long, green (brown near the beak), veined, subsessile, abruptly narrowed into an elongated beak 0.9–1.2 mm long. However, the C113 specimen somewhat differs from *C. jacutica* by having scarcely emarginate, more rarely bidentate-emarginate beak (in contrast to the one with short teeth in *C. jacutica*) and by the presence of flowers with 2 and 3 stigmas. Parent species of this specimen cannot be identified precisely based on the studied sequences. However, we believe that *C. saxatilis* subsp. *laxa* is the most probable parent species of this hybrid, because 2 stigmas and a scarcely emarginate beak are characteristic for this species. In addition, *C. saxatilis* subsp. *laxa* is the only representative of the clade A that has been reported from the region where the C133 specimen was collected. This specimen appears to be fertile: utricules contain full-developed fruits and opened anthers with remnants of pollen.

No interspecific hybrids between the members of clade B were found, although they could be easily detected using the *hsp90i* sequence. Lack of sequence variation within clade A impedes detection of hybrids.

Phylogeny of sect. *Vesicariae*

We constructed phylogenetic trees for all four markers separately, for concatenated nuclear (ITS2 + *hsp90i*) and plastid (*atpF-H* + *matk*) sequences (Fig. 1), and for the combined set of all four sequences (Fig. 2) (only for the specimens for which all corresponding sequence markers were sequenced). Phylogenetic trees constructed using both nuclear (ITS2 + *hsp90i*) and plastid (*atpF-H* + *matk*) sequence sets confirm the existence of the clade A (*C. vesicata*, *C. vesicaria*, *C. saxatilis* subsp. *laxa*, and both subspecies of *C. pamirica*) group. On the plastid tree, clade B (*C. mollissima*, *C. jacutica*, *C. rotundata* and *C. rostrata*) is split into two parts, one of which also includes *C. rhynchophysa*; however, we should note that this is based on only a single substitution in the *matK* gene.

Carex rhynchophysa differs considerably morphologically from other representatives of the section *Vesicariae*: it has a large number of staminate spikelets (3–7, in contrast to 1–4

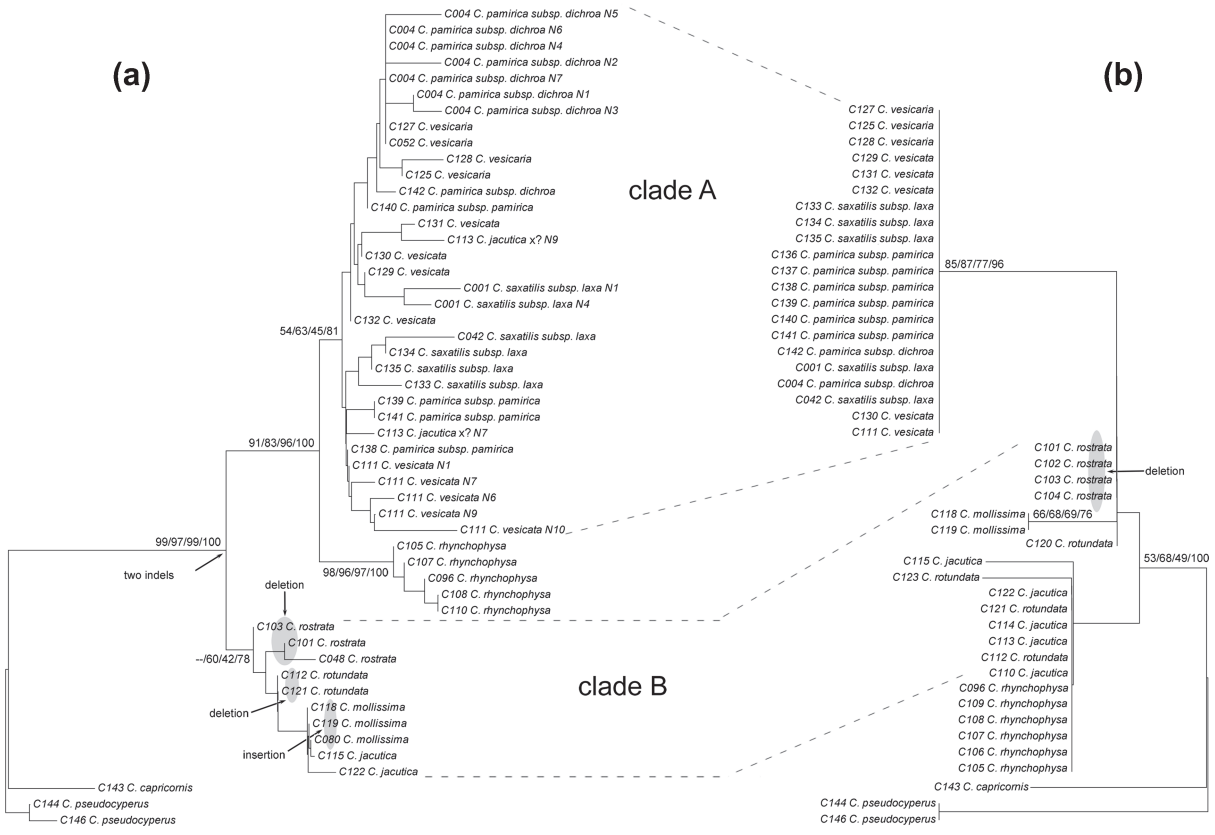

Figure 1. Phylogenetic trees of (a) *hsp90i* + ITS2 and (b) *matK* + *atpF-H* concatenated sequences constructed using ME algorithm. Numbers on branches indicate bootstrap support for ME/MP/ML algorithms/Bayesian posterior probabilities.

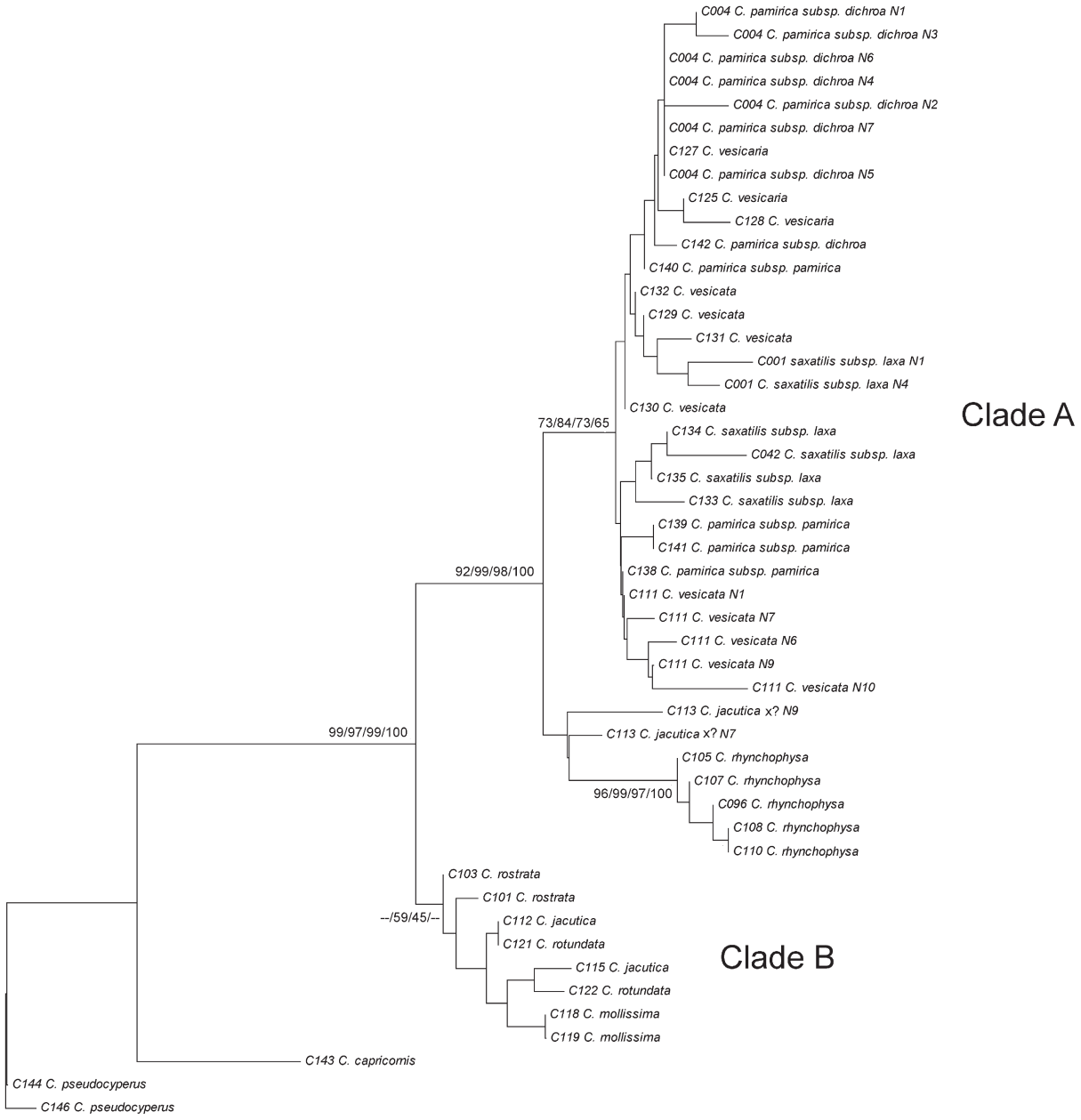

Figure 2. Phylogenetic tree constructed using the combined set of all four sequences used in this study (*hsp90i* + ITS2 + *matK* + *atpF-H*).

in other species of the section), and big pistillate spikelets with high number of flowers (Egorova 1999). Based on these differences, it is often considered to be the most primitive representative of this section (Egorova 1999). Sequence data conform with this special position of *C. rhynchophysa* within the section: based on plastid sequences it falls into clade B, but according to *hsp90i* sequences it forms a separate clade that is close to clade A. This may suggest that *C. rhynchophysa* might have arisen as the result of ancient intraspecific hybridization. According to Ford et al. (1993), *C. rhynchophysa* may be a synonym of *C. utriculata*; however, this notion was not supported by Egorova (1999). Unfortunately, there are no sequences of this species in GenBank.

Clade A includes *C. vesicaria*, *C. vesicata*, *C. saxatilis* subsp. *laxa*, and two subspecies of *C. pamirica*. The plastid sequences of the members of this clade are identical; and *hsp90i* sequences found only minor differences among them. *Carex saxatilis* subsp. *saxatilis matK* and *atpF-H* sequences from GenBank also fall in this group. According to allozyme studies by Ford et al. (1993), *C. vesicaria* and *C. saxatilis* subsp. *saxatilis* fall in a group separate from *C. utriculata*, *C. membranaceae*, *C. rostrata* and *C. rotundata*, although *C. saxatilis* belongs to the short-beaked group, and *C. vesicaria* to the long-beaked group.

Clade B includes *C. rostrata*, *C. mollissima*, *C. jacutica* and *C. rotundata*. *Carex rostrata*, *C. rotundata* and *C. mollissima* have characteristic indels that make them easily identifiable and distinguish them from other species of the section. As expected, *C. membranacea matK* and *atpF-H* sequences from GenBank also fall in this group.

In summary, we identified two groups within the section *Vesicariae*; this division is based on molecular data and correlates with the nature of the transition of the utricle into the beak, which is gradual in clade A and abrupt in clade B. Egorova (1999) listed this utricle character among the most constant diagnostic characters, in contrast to form, size, color, and beak length. This division is in good accordance with the results of allozyme studies by Ford et al. (1991, 1993). These groups are also in accordance with the taxonomic system of Kreczetowicz (1935), except for the position of *C. membranacea* and *C. mollissima*. We conclude that phylogenetic analysis performed using nuclear and plastid sequence markers allowed us to clarify relationships between the species of the section *Vesicariae* and may become the basis for recognition of subsectional taxa within this section.

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